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Validation and application of the 3D human reconstructed skin micronucleus assay (RSMN) using the EpiDerm™ tissue to the safety assessment of cosmetics ingredients

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Abstract

The Reconstructed Skin Micronucleus assay (RSMN) using EpiDerm™, is a more realistic model for evaluating the genotoxic potential of dermally applied chemicals. The assay exhibits good transferability and inter- and intra-laboratory reproducibility. In Phase 3, the assay protocol was modified (e.g. extension of the treatment from 48h to 72h) and an additional 12 coded chemicals were subsequently tested. There was an excellent overall specificity (87%) with only few mispredictions. Six true positive chemicals were initially negative using a 48h dosing regimen but were positive when tested in a 72h dosing regimen. The inclusion of a 72h dosing regimen increased the sensitivity to 80%. Two of the 3 chemicals missed by the 72h regimen (2-AAF and $CaCl_2$), are Ames positive and were picked up by the 3D skin Comet assay. These data support the use of the RSMN assay for follow-up of positive results from standard *in vitro* assays and therefore removing the need for *in vivo* follow-up testing.

Introduction

Regulatory restrictions on animal use have increased the reliance of risk assessors and regulators on *in vitro* test systems. Ideally, tissue-based assays could replace the animal studies as follow-up tools to verify results from standard *in vitro* assays. The RSMN assay combines the EpiDerm™ 3D reconstructed skin (RS) model with the micronucleus (MN) assay to provide a more realistic model for evaluating the genotoxic potential of dermally applied chemicals or products, such as cosmetics. This assay is expected to be used as a follow-up for positive results from the standard in vitro genotoxicity battery[1]. Cosmetics Europe has funded the establishment and evaluation of the RSMN assay and shown it to have good transferability, inter- ad intra-laboratory reproducibility in validation studies[2,3]. In Phase 3, the predictive capacity of the assay was explored and the sensitivity observed with the standard 48h treatment protocol was insufficient (65%) which led to assay modifications (e.g. extension of the treatment from 48h to 72h). So-called 'bridging studies with 12 coded chemicals were performed to evaluate the performance of the modified protocol which included a 72h treatment as verification of negative or equivocal results in the initial 48h treatment.

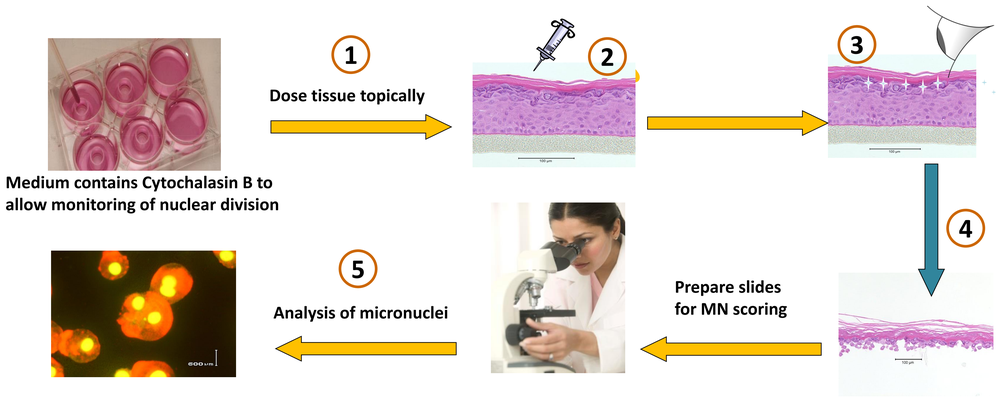
Methods

A detailed protocol for the 3D skin MN assay was published, together with a harmonized scoring atlas for micronuclei[4]. An overview of the method and the modified criteria **(in bold)** is shown below.

1. EpiDerm™ models are treated topically with test compound.
2. Dose at 24h intervals (48h or **72h total**)
3. **Precipitation at the beginning and the end of the treatment period is noted.**
4. Keratinocytes are released by trypsinization
5. Micronuclei in binucleated cells are counted by visual scoring.

Additional criteria were applied:

- **The lowest precipitating concentration was the highest dose for the evaluation of micronuclei**
- **A negative outcome in the first 48h experiment was verified by additional 72h experiments. If the results were positive at 72h, the overall call was positive**



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Results

- The validation results are summarized in the following table:

Parameter	Phase 1-3 testing	+ Bridging studies
Sensitivity	62.5%	88.0%
Specificity	95.2%	87.0%
Overall concordance	86.2%	89.5%

- Overall, the data demonstrate and excellent overall specificity (87%) in the RSMN assay with only few mispredictions: diclofenac (3/3 labs), phenanthrene (1/4), resorcinol (1/2) and curcumin (1/1 lab - also positive in all other in vitro assays).
- Considering sensitivity, there were 6 true positive chemicals that were initially negative using a 48h dosing regimen but were positive when tested in a 72h dosing regimen. The inclusion of a 72h dosing regimen increased the sensitivity to 80%.
- Two out of the three chemicals that were missed by the 72h regimens, totally or partially (2-AAF and CaCl2), have also been tested in the process of validation of the 3D skin Comet assay and were found genotoxic in this assay. This suggest that the calculated sensitivity presents a conservative estimate of the sensitivity of tissue-based genotoxicity assays since both 2-AAF and $CaCl_2$ are Ames positive compounds which would have been be picked up if tested in an endpoint-driven approach, increasing the sensitivity to 92%.

Conclusions

- **An international validation study with 38 coded chemicals shows a high sensitivity (80%) and specificity (87%) for the prediction of in vivo genotoxicity outcomes**
- **Two of the compounds missed are Ames positive and would be picked up by the 3D skin Comet assay, which increases the sensitivity to 92%**
- **The data supports the use of the human 3D skin-based genotoxicity assays for follow-up of unfavourable results from standard in vitro assays (e.g., Ames, micronucleus) and therefore is a direct replacement of in vivo follow-up testing**

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References: 1-Pfuhler *et al*. Reg Pharm Tox 2010; 57(2-3):315-324; 2-Hu *et al*. Mutat Res. 2009, 673(2):92-9; 3-Aardema *et al*. Mutat Res. 2010, 701(2):123-31; 4-Dahl *et al*. Mutat Res. 2011 28;720(1-2):42-52..